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Analysis of *Escherichia coli* Global Gene Expression Profiles in Response to Overexpression and Deletion of CspC and CspE

Sangita Phadtare,[1]* Vasisht Tadigotla,[2] Weon-Hye Shin,[3] Anirvan Sengupta,[2,4] and Konstantin Severinov[5]

Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854[1]; BioMaPS Institute for Quantitative Biology, Rutgers, The State University of New Jersey, 110 Frelinghuysen Road, Piscataway, New Jersey 08854[2]; Korea Intellectual Property Office, Gov. Complex Darjeon Bldg. 4, Dunsan-dong, Seo-gu, 302-701, Daejeon City, Korea[3]; Department of Physics and Astronomy, Rutgers, The State University of New Jersey, 136 Frelinghuysen Road, Piscataway, New Jersey 08854[4]; and Waksman Institute, Department of Biochemistry and Molecular Biology, Rutgers, The State University of New Jersey, 190 Frelinghuysen Road, Piscataway, New Jersey 08854[5]

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The *Escherichia coli* cold shock protein CspA family consists of nine proteins (CspA to CspI), of which two, CspE and CspC, are constitutively produced at 37°C and are involved in regulation of expression of genes encoding stress response proteins but can also perform an essential function during cold acclimation. In this study, we analyzed global transcript profiles of cells lacking *cspE* and *cspC* as well as cells individually overexpressing these proteins or a CspE mutant that is unable to melt nucleic acids and is defective in cold acclimation. The analysis reveals sets of genes whose expression (i) is regulated by CspC and CspE at physiological temperature or cold shock conditions and (ii) depends on the nucleic acid melting function of CspE. Bioinformatic analysis of the latter group reveals that many of those genes contain promoter-proximal sequences that can block transcript elongation and may be targeted by the nucleic acid melting function of CspE.

When exponentially growing *Escherichia coli* cells are transferred to 15°C, there is a growth lag period characterized by a transient high-level induction of cold shock proteins, which occurs at a background of dramatic reduction in the total protein synthesis. The major cold shock protein, CspA, is one of the nine homologous proteins (CspA through CspI) of *E. coli*. Among the CspA family proteins, only CspA, CspB, CspG, and CspI are cold shock inducible; CspC and CspE are constitutively produced at 37°C, and CspD is induced by nutrition deprivation and at stationary phase at 37°C (for review, see references 3, 10, and 19). Cells harboring single, double, or triple deletions of cold-inducible *csp* genes grow at low temperature, suggesting that none of the CspA homologues are singularly responsible for cold shock adaptation (20). CspE is induced by cold shock in a triple deletion mutant (Δ*cspA* Δ*cspB* Δ*cspG*) while a quadruple deletion strain (Δ*cspA*Δ*cspB*Δ*cspG*Δ*cspE*) is cold sensitive, suggesting that the cold acclimation function(s) of CspA proteins is redundant and that CspE, which is normally not induced at cold shock, can perform essential cold acclimation function(s). In fact, the quadruple deletion can be complemented for growth at low temperature by overproduction of any one of the nine *cspA* family genes (except *cspD*) (20).

Among the nine Csp proteins, CspC and CspE stand out, as they seem to function both at the physiological temperature and during cold shock. Several diverse functions, including, for example, chromosome decondensation and regulation of the PNPase activity, have been assigned to CspE (4, 6, 8). We showed previously that CspC and CspE upregulate the expression of a gene encoding a global stress response regulator RpoS (RNA polymerase σ^S subunit) (13). The upregulation is caused by the *rpoS* message stabilization. Expression of genes such as *dps*, *katG*, *proP*, and *uspA*, a gene encoding a protein that is induced by numerous stresses, also depends on CspC and CspE, though the effect may be indirect since expression of these genes except *uspA* is known to be RpoS dependent (13, 15).

CspA, CspC, and CspE bind RNA and single-stranded DNA with low affinity and low specificity (9, 14). The binding leads to energy-independent melting and/or destabilization of nucleic acid secondary structure elements (2, 15). Disruption of nucleic acid melting activity of CspE by mutation abolishes its cold acclimation function(s) (15). Presumably, the nucleic acid melting function of Csp proteins is essential at cold shock, a condition that should impede transcription and translation through stabilization of secondary structures in RNA (2, 5, 9, 15, 16).

Genes whose expression may depend on the RNA chaperoning function of CspA and its homologues were revealed by comparing cold shock global transcript profiles of wild-type and Δ*cspA* Δ*cspB* Δ*cspG* Δ*cspE* quadruple deletion strain (12). As expected, deletion of the four *csp* genes prominently affected genes that are normally transiently induced during cold acclimation (12). However, this analysis, though informative, could not dissect the observed effects with respect to known biochemical activities of Csp proteins (nucleic acid binding versus nucleic acid melting).

In this report, we performed comprehensive transcription profiling of 37°C-grown cells overexpressing CspC or CspE, or lacking both CspE and CspC. We also analyzed cells overexpressing a CspE mutant that binds nucleic acids normally but is unable to

* Corresponding author. Mailing address: Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Phone: (732) 235-4116. Fax: (732) 235-4559. E-mail: phadtasa@umdnj.edu.

TABLE 1. Genes changed in response to overproduction and deletion of CspC and CspE[a]

Gene	Gene product and/or function	CspE/wt (37°C)	CspC/wt (37°C)	Δ*cspC/E*/wt (37°C)	Wild-type 15°C/37°C ratio[b]	Mutant/wild type ratio[b] (15°C, 1 h)	CspEF30R/wt (37°C)
Genes whose expression requires CspC/E							
Membrane synthesis/function							
entC	Isochorismate synthase	4.3 ± 0.6	5 ± 0.6	0.47 ± 0.05	NC	0.71 ± 0.15	1.2 ± 0.1
malE	Maltose-binding protein precursor	3.99 ± 0.58	4.1 ± 0.4	0.48 ± 0.5	9 ± 0.5	0.03 ± 0.01	0.68 ± 0.02
malK	MalK	3.9 ± 0.7	4.7 ± 0.02	0.49 ± 0.5	10 ± 0.5	0.04 ± 0.01	0.89 ± 0.02
Cell metabolism (*citX*)	CitX protein	3.88 ± 0.14	6.66 ± 0.7	0.69 ± 0.05	NC	0.59 ± 0.02	1.6 ± 0.2
Diverse functions							
dps	DNA-binding protein Dps	4 ± 0.3	5.57 ± 0.9	0.5 ± 0.08	10 ± 1.3	0.05 ± 0.01	0.77 ± 0.05
katG	Catalase hydroperoxidase I	4 ± 0.5	4.5 ± 0.6	0.5 ± 0.07	17 ± 0.5	0.44 ± 0.15	0.49 ± 0.06
mopA	GroEL protein	3.5 ± 0.2	3.5 ± 0.1	0.48 ± 0.05	9.3 ± 0.88	0.13 ± 0.01	0.87 ± 0.03
mopB	GroES protein	3.5 ± 0.2	3.45 ± 0.1	0.43 ± 0.05	8 ± 1	0.18 ± 0.04	0.83 ± 0.1
rpoS	RNA polymerase sigma factor RpoS	4.5 ± 0.8	3 ± 0.1	0.25 ± 0.05	2.7 ± 0.3	0.3 ± 0.01	3.4 ± 0.4
uspA	Universal stress protein	3.67 ± 0.2	3.6 ± 0.2	0.4 ± 0.02	2.7 ± 0.1	0.44 ± 0.04	3.0 ± 0.2
Genes whose expression require Csp proteins at 15°C but not at 37°C							
Membrane synthesis/function							
csgC	Putative curli production protein	3.88 ± 0.6	2 ± 0.2	NC	NC	0.75 ± 0.01	0.56 ± 0.1
hdeA	HdeA protein	NC	3.9 ± 0.015	NC	NC	0.34 ± 0.04	1.0 ± 0.01
hdeB	10K-1 protein precursor	NC	3.6 ± 0.05	NC	NC	0.48 ± 0.08	1.0 ± 0.0
mmuP	*S*-Methylmethionine permease	4.23 ± 0.75	1.7 ± 0.3	NC	NC	0.015 ± 0	0.71 ± 0.08
tar	Methyl-accepting chemotaxis protein	7.61 ± 0.14	7.5 ± 0.5	NC	11.1 ± 0.85	0.8 ± 0.2	0.71 ± 0.02
tnaB	Low-affinity tryptophan permease	6.89 ± 0.62	7.04 ± 0.2	NC	2.9 ± 0.2	0.07 ± 0.02	0.65 ± 0.2
Cell metabolism							
fruB	Phosphotransferase system fructose-specific component	4.6 ± 1.3	4.6 ± 1.2	NC	7 ± 0.16	0.16 ± 0.03	0.19 ± 0.0
fruK	1-Phosphofructokinase	3.2 ± 0.08	3.8 ± 0.2	NC	6.5 ± 0.75	0.18 ± 0.015	0.29 ± 0.09
fumB	Fumarate hydratase	12.1 ± 1.47	12.47 ± 1.2	NC	9.3 ± 0.79	0.65 ± 0.25	0.34 ± 0.08
pyrB	Aspartate carbamoyltransferase	2.1 ± 0.4	8.5 ± 0.5	NC	NC	0.27 ± 0.08	0.31 ± 0.02
pyrI	Aspartate carbamoyltransferase	3.34 ± 0.16	4.2 ± 0.3	NC	NC	0.9 ± 0.03	0.30 ± 0.01
tnaA	Tryptophanase	5 ± 0.7	6 ± 0.8	NC	2.8 ± 0.2	0.02 ± 0.01	1.18 ± 0.07
treC	Trehalose-6-phosphate hydrolase	4.8 ± 1.08	NC	NC	6 ± 1	0.12 ± 0.01	0.70 ± 0.04
Diverse functions (*rbsK*)	Ribokinase	3.6 ± 0.2	3.0 ± 0.5	NC	5.1 ± 0.34	0.19 ± 0.04	0.67 ± 0.05
Genes whose expression is upregulated by Csp proteins at 37°C, but does not depend on Csp proteins at high/low temperature							
Membrane synthesis/function							
cheW	Chemotaxis protein	4 ± 0.5	2.32 ± 0.2	NC	3.5 ± 0.05	NC	1.02 ± 0.01
dcuB	Anaerobic C_4 dicarboxylate transporter	8.8 ± 0.53	6.8 ± 0.2	NC	7.4 ± 0.36	NC	1.0 ± 0.2
feoA	Iron(II) transport protein FeoA	1.62 ± 0.1	6 ± 0.5	NC	NC	NC	0.88 ± 0.07
feoB	Iron(II) transport protein FeoB	1.7 ± 0.1	6 ± 0.6	NC	NC	NC	0.84 ± 0.01
flhA	FlhA protein	3.96 ± 0.69	2.44 ± 0.2	NC	0.76 ± 0.04	NC	1.13 ± 0.01
flhB	FlhB protein	4.56 ± 0.23	2.1 ± 0.2	NC	1.38 ± 0.12	NC	0.83 ± 0.06
motB	Chemotaxis MotB protein	3.68 ± 0.09	1.35 ± 0.18	NC	0.81 ± 0.07	NC	0.78 ± 0.15
phnC	PhnC-phosphonates transport protein	5.24 ± 1.2	2.5 ± 0.1	NC	NC	2.1 ± 0.2	1.06 ± 0.09
tap	Methyl-accepting chemotaxis protein	9.6 ± 0.7	4.73 ± 0.3	NC	4.2 ± 0.12	NC	0.97 ± 0.07
Cell metabolism							
adiY	Probable regulatory protein, AdiY	8.4 ± 3.2	3.86 ± 0.2	NC	NC	2.0 ± 0.4	0.9 ± 0.12
evgA	EvgA regulatory protein	3.7 ± 0.7	5.62 ± 0.5	NC	NC	1.32 ± 0.16	0.9 ± 0.05
hyaA-F	Hydrogenase, HyaC/D/E/F proteins	1.5 ± 0.1	5–12 ± 0.7	NC	NC	NC	0.8 ± 0.05
wrbA	Trp repressor binding protein	0.3 ± 0.02	7 ± 0.5	NC	NC	NC	0.82 ± 0.14
Diverse functions							
alpA	Prophage-cp4-57-regulatory protein AlpA	4.5 ± 0.9	1.7 ± 0.1	NC	NC	NC	0.9 ± 0.1
appY	M5 polypeptide	5.2 ± 0.6	3.99 ± 0.4	NC	NC	NC	0.77 ± 0.3

cchB	CchB protein	4.26 ± 0.4	NC	NC	NC	NC	0.9 ± 0.1
fdrA	Multicopy suppressor, FdrA	4.8 ± 0.8	NC	NC	NC	NC	1 ± 0.02
slp	Slp protein	NC	12.6 ± 2	NC	NC	NC	1.0 ± 0.03
Genes which respond miscellaneously to Csp proteins							
Membrane synthesis/function							
exbB	Biopolymer transport protein ExbB	2.25 ± 0.1	3.62 ± 0.16	0.7 ± 0.1	0.4 ± 0.06	3.5 ± 0.5	1.2 ± 0.1
fecI	FecI-iron citrate transport	3.65 ± 0.28	4.1 ± 0.5	0.77 ± 0.05	NC	3.0 ± 0.5	1.12 ± 0.09
fepE	FepE protein	4.42 ± 0.6	1.8 ± 0.1	0.66 ± 0.05	NC	1.35 ± 0.05	1.1 ± 0.02
fepG	FepG protein	3 ± 0.2	1.8 ± 0.2	0.58 ± 0.04	NC	1.3 ± 0.08	1.3 ± 0.08
fes	Fes protein	7.2 ± 1.4	7 ± 1.5	0.92 ± 0.06	NC	2.5 ± 0.8	0.88 ± 0.01
fhuA	Ferrichrome-iron receptor precursor	5.79 ± 0.36	2.6 ± 0.2	NC	NC	3.9 ± 0.4	1.02 ± 0.02
fhuD	Ferrichrome-iron transport protein	3.21 ± 0.5	NC	0.92 ± 0.06	NC	3.0 ± 0.5	1.02 ± 0.09
fhuF	Ferric hydroxamate transport protein	6 ± 1	3 ± 1.2	0.64 ± 0.05	NC	7 ± 0.2	1.24 ± 0.2
sfa	Sfa protein	4.41 ± 0.51	3.2 ± 0.2	0.73 ± 0.06	0.43 ± 0.03	NC	0.80 ± 0.13
Cell metabolism (*bioF*)	Biotin synthesis protein	5.04 ± 0.97	NC	0.7 ± 0.04	NC	NC	1.04 ± 0.1

[a] CspE/wt, gene upregulation by CspE overexpression (ratio compared to wild type); CspC/wt, gene upregulation by CspC overexpression (ratio compared to wild type); Δ*cspC/E*/wt, expression change in the Δ*cspC* Δ*cspE* double deletion mutant; CspEF30R/wt, transcript levels in cells overproducing melting-deficient mutant of CspE ($CspE^{F30R}$). Ratios averaged from three independent sets of experiments are shown with standard deviation values. NC indicates no change, or a ratio of 1 or very close to 1. Ratios above 1 indicate induction and those below 1 indicate repression. However, for the column showing results obtained with $CspE^{F30R}$ overproduction, actual values are given even if the ratios are 1 or close to 1 in order to allow quantitative comparisons with the "CspE/wt" column.

[b] For comparison, changes in expression of these genes observed previously (12) during global transcript profiling of wild-type or cold-sensitive *csp* quadruple deletion cells undergoing cold shock are presented. Note that in the "Wild type 15°C/37°C ratio" column, transcript levels at 15°C are compared with those at 37°C in the wild-type cells, while in the "Mutant/wild type ratio (15°C, 1 h)" column, cold-shocked quadruple deletion cells and the cold-shocked wild-type cells are compared to each other.

perform nucleic acid melting to determine the genes whose expression depends on the melting function of CspE.

MATERIALS AND METHODS

Bacterial strains. *E. coli* wild-type strain JM83 [F^- *ara*Δ(*lac-proAB*) *rpsL*(Str^r)] (21) and its *cspC-cspE* double deletion strain (13) were grown in M9 medium supplemented with glucose (0.02 to 0.4%) and 0.4% Casamino Acids. Ampicillin (50 μg ml^{-1}) was supplemented as required. The IPTG (isopropyl β-D-thiogalactopyranoside)-inducible pINIII plasmid and the pINIII-*cspC*, pINIII-*cspE*, and pINIII*cspE*-F30R expression vectors were described previously (2, 13).

RNA isolation. *E. coli* cells grown overnight in M9 medium at 37°C were diluted into fresh medium. Cells of the JM83 wild type and its *cspC-cspE* double deletion strain were grown at 37°C to exponential phase (optical density at 600 nm [OD_{600}] of 0.8) and were harvested. To examine the effect of overexpression of CspC and CspE, the pINIII, pINIII-*cspC*, and pINIII-*cspE* plasmids were transformed into the JM83 strain. The exponentially growing cells at an OD_{600} of 0.5 at 37°C were induced with 1 mM IPTG for 30 min. The cells were then harvested for RNA isolation. The total RNA was extracted by the hot phenol method described previously (17). It was further purified by RNeasy minikit (QIAGEN) and was then treated with DNase I, followed by phenol-chloroform treatment and ethanol precipitation. It was quantified by measuring absorbance at 260 nm. The purity of RNA was confirmed by agarose gel electrophoresis.

DNA microarray analysis. The mRNAs were converted to cDNAs with coincident labeling with Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia). Random hexamer $pd(N)_6$ (Amersham Pharmacia) was used as a primer. We used IntelliGene *E. coli* CHIP, version 2 (Takara Bio. Inc., Japan), which represents *E. coli* K-12 W3110 open reading frames. The analysis of the density of each spot and calculation of expression ratio for each spot was carried out by using the analyzing software Imagine, version 4 (catalog no. BD001; Takara). For the adjustment of signals between Cy3 and Cy5, the DNA chip included internal controls.

Primer extension. The primer extension and the deoxyoligonucleotides used for detection of *cspA*, *katG*, *mopB*, *rpoS*, and *uspA* were described previously (12, 13). The deoxyoligonucleotide used for detection of *rbsK* (7) corresponds to the region from codons 13 through 6 of *rbsK*. The primers were labeled with [γ-^{32}P]ATP (Dupont-New England Nuclear) by using T4 polynucleotide kinase (Gibco BRL). Primer extension was carried out with 5 μg of RNA at 42°C for 1 h in a final reaction volume of 10 μl, with 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 0.4 pmol of ^{32}P-labeled primer, 0.5 mM each of the dNTPs, 10 U RNase inhibitor (Boehringer Mannheim), and 6.25 U reverse transcriptase (Boehringer Mannheim). The products were analyzed on a 6% polyacrylamide gel under denaturing conditions. Quantitation of primer extension products was carried out by direct radioactive measurements.

Radioactive labeling of the cells and two-dimensional gel electrophoresis. Cells (JM83 wild-type and its *cspC-cspE* double deletion strain) were grown in M9 medium supplemented with glucose, 19 amino acids (without methionine), and thiamine at 37°C to an OD_{600} of 0.5. Portions (one-milliliter) of the cultures were labeled with [^{35}S]methionine (1,092 Ci mol^{-1}, 53 Ci ml^{-1}; Amersham) for 5 min and then were chased for 3 min by adding nonradioactive methionine to a final concentration of 0.2 M. Cell lysates were prepared and were analyzed by two-dimensional gel electrophoresis (18).

In vitro transcription. The in vitro transcription was carried out as described previously in solution (11, 15). CspE was purified as described previously (15). A DNA template containing the T7A1 promoter fused to a 100-nucleotide (nt) sequence containing presumed promoter proximal terminator for *tnaCAB* (5′-GCCGGTTTCACTGGCAAACTTAAAAGCGATGTACAGCATCGCGAAG AAATACGATATTCCGGTGGTAAGGACTCCGCGCGCTTTGCTGAAAA CGCCTATTT-3′) was used. The elongation complexes stalled at position +20 were prepared in transcription buffer (40 mM KCl, 40 mM Tris-HCl, pH 7.9, and 10 mM $MgCl_2$) containing 20 nM of T7 A1 promoter-DNA fragments, 40 nM His-tagged RNA polymerase (RNAP), and 0.5 mM ApU. Reaction mixtures were incubated at 37°C for 15 min to form the open complexes and then were transferred to room temperature. Concentrations of 50 μM ATP, 50 μM GTP, and 2.5 μM [α-^{32}P]CTP (300 Ci/mmol) were added. After 10 min of incubation, the reaction mixtures were supplemented with Csp proteins (1.5 μg) and NTPs (250 μM) and were incubated at room temperature for 10 min. After the transcription reactions, 20 mM EDTA and 10 mg/ml heparin was added to the reaction mixtures to avoid nonspecific retardation of RNA in the gel. The reactions were terminated by formamide-containing loading buffer. The products were analyzed by urea-polyacrylamide gel electrophoresis (7 M urea–10% polyacrylamide) followed by autoradiography and phosphorimager analysis. *galP1*pDW304- and lambda promoter-containing DNAs were used as control templates to mark the positions of the runoff and terminated products.

RESULTS AND DISCUSSION

The goal of this work was to identify all of the *E. coli* open reading frames whose mRNAs undergo a significant change in abundance in response to changes in the levels of CspC and CspE. The following conditions were investigated: (i) cells lacking both *cspE* and *cspC* genes and growing at 37°C, (ii) wild-type cells growing at 37°C and overproducing CspE or CspC from plasmids, and (iii) wild-type cells growing at 37°C and overproducing a CspE mutant that is unable to melt nucleic acids.

Total cellular RNAs were isolated and used to generate probes that were hybridized to DNA arrays, and the results were quantified as described in Materials and Methods. Each microarray experiment was independently repeated three times and label swaps were used to ensure consistency. The cell density of the control and test cells for each set was the same; thus, the observed changes in mRNA levels were not due to differences in cell densities. For overexpression of Csp proteins, appropriate IPTG-inducible pINIII-vector based plasmids were used. Cells carrying the pINIII vector served as control for these sets. Our previous studies using a proteomic approach showed that certain genes are regulated by both CspC and CspE (13). Therefore, a Δ*cspC* Δ*cspE* double deletion strain rather than single deletion strains was used to test the effect of the absence of these Csp proteins. Wild-type *E. coli* JM83 cells were used as a control for this set of microarrays.

The results of global transcript profiling are shown in Table 1. Only genes whose expression levels were upregulated by at least a factor of three compared to the control set are included in the table. The use of replicate datasets allowed us to estimate that the probability of a threefold difference in mRNA levels arising from random fluctuations was less than 0.12%, corresponding to a confidence interval of 99.88%. Thus, the chosen cutoff value is stringent and changes in expression beyond the cutoff are highly statistically significant.

The differences in mRNA levels that emerge from the array data are reliable, as (i) reproducible values were obtained in multiple, independent experiments, (ii) genes such as *uspA*, *rpoS*, *dps*, *katG*, and *mopA*, whose upregulation upon overproduction of either CspC or CspE was previously detected by a proteomic approach (13), showed induction in the DNA microarray analysis, and (iii) for each randomly chosen gene, changes in expression detected by microarrays are confirmed by primer extension (Fig. 1) and Northern blot analysis (data not shown).

Previously, we reported that CspE overexpression at 37°C leads to increased expression of several promoter-distal genes of the *metY-rpsO* operon due to transcription antitermination (2). Although the products of these genes-NusA, IF2, RbfA, and PNPase-are known to be involved in cold acclimation of cells, our previous cold shock microarray data (12) did not show increased mRNA abundance of *nusA*, *infB*, *rbfA*, and *pnp* genes of the operon. The reason for this result remains unexplained. In the present study, transcript levels of the *metY-rpsO* operon's *nusA*, *pnp*, and *yhbC* genes were moderately changed upon overproduction of CspC and CspE (1.5- to 2-fold increase). The reason for such a small change, which is in contrast to the drastic increase detected by Northern blot analysis (2) is not known, and may be due to an inherent shortcoming of the array system used.

The CspE/CspC-responsive genes in Table 1 are categorized into four groups, as explained below. In each group, genes are further categorized based on their cellular functions (i.e., membrane synthesis and function, cellular metabolism, and various "diverse" functions).

The expression of genes in the first group is increased upon CspE/CspC overproduction and is decreased in the Δ*cspC* Δ*cspE* double deletion strain. At cold shock, group 1 genes are strongly induced (with the exception of *entC* and *citX*) and all are downregulated in the Δ*cspA* Δ*cspB* Δ*cspA* Δ*cspE* strain. Thus, CspE and/or CspC normally present in *E. coli* exponentially growing at 37°C are required for optimal expression of these genes and their upregulation at cold shock is due, directly or indirectly, to cold-induced Csp overproduction. The cold shock data also suggest that most group 1 genes are activated not only by CspE and/or CspC but also by cold-inducible Csp proteins. In this regard, *entC* and *citX* are unique as they may specifically respond to CspE/CspC overproduction.

Upregulation of some of group 1 genes upon CspE/CspC overproduction could be indirect and due to increased levels of RpoS. For example, expression of *dps*, a group 1 gene, is low in the *rpoS* mutant strain (13). Cells overproducing melting-deficient CspEF30R failed to upregulate all group 1 genes with the exception of *rpoS* and *uspA*, two genes that were previously shown to require CspE binding for their mRNA stabilization and do not depend on its nucleic acid melting activity (13, 15). The fact that upregulation of these two genes in response to mutant CspE overproduction is detected by microarray demonstrates that the mutant CspE is stably produced in the cells, but its regulatory effect is restricted to these genes. Thus, most group 1 genes are upregulated due to nucleic acid melting/transcription antitermination activity of Csp proteins. These group 1 genes include *dps* and *katG*, which are both RpoS-regulated. This result implies that for optimal expression, these genes require both RpoS and the melting function of Csp proteins.

The expression of genes in the second group is increased upon CspE or CspC overproduction but is not affected by deletion of *cspE* and *cspC*. At cold shock, approximately half of group 2 genes are upregulated, and many are downregulated in the quadruple deletion strain. Therefore, group 2 genes such as *hdeA*, *hdeB*, *mmuP*, and *pyrB* though not cold shock-inducible, depend on Csp proteins for their basal expression levels at low temperature, but not at 37°C. Certain genes such as *csgC*, *tar*, and *pyrI* respond only marginally to the deletion of *csp* genes at low temperature. Three of the group 2 genes show differential response to CspC/CspE overproduction. At 37°C, *hdeA* and *hdeB* are strongly induced when CspC is overproduced but are unaffected by overproduction of wild-type or mutant CspE. These genes are not induced by cold shock but are downregulated in the quadruple mutant from which CspE is absent. Therefore, the optimal expression of these genes may specifically require CspE, but only at low temperature. The *treC* gene expression is strongly induced by CspE but not by CspC overproduction and is also cold shock-inducible and is decreased in the quadruple mutant.

The expression of genes in the third group is not downregulated in the absence of Csp proteins at either 37°C or at cold shock. However, their upregulation upon overproduction of Csp proteins at 37°C is due to Csp's increased nucleic acid melting, for no upregulation is detected in cells overexpressing

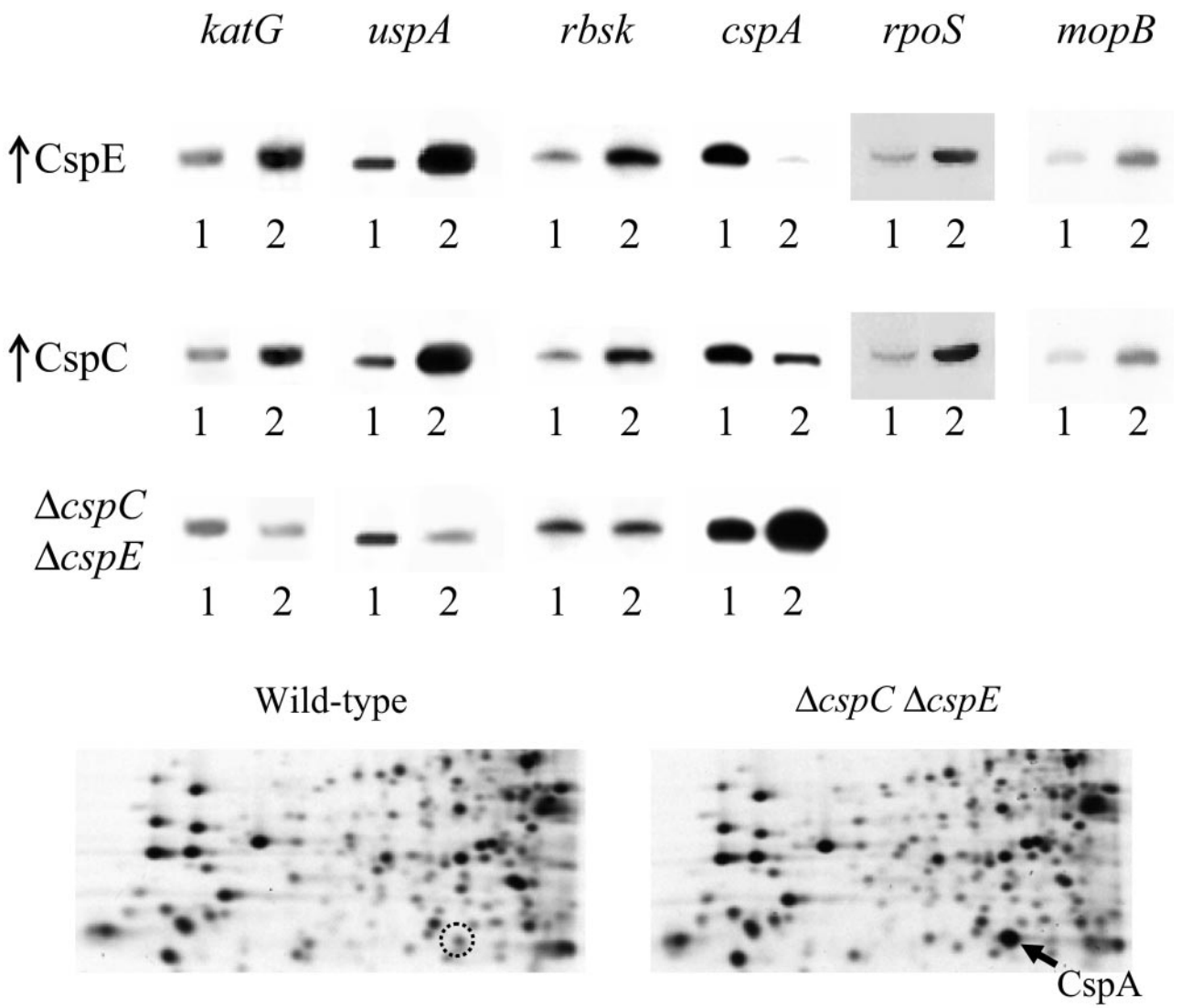

FIG. 1. Effect of overexpression and deletion of CspC and CspE on the levels of mRNAs. In the upper panel, primer extension results are shown. Total RNA was extracted by the hot phenol method as described in the text, and primer extension was carried out with deoxyoligonucleotides corresponding to the respective genes. Lanes 1 and 2 in each case represent mRNAs isolated from control and test cells, respectively. Cells carrying pINIII vector was used as control for cells overexpressing CspC or CspE. JM83 cells were used as control for Δ*cspC* Δ*cspE* strain. In the lower panel, part of a gel analyzed by two-dimensional gel electrophoresis is shown. The JM83 and its Δ*cspC* Δ*cspE* strain cells were labeled with [^{35}S]methionine and the protein synthesis patterns were compared by two-dimensional gel electrophoresis. The first dimension (isoelectric focusing) gel was carried out between the range of pH 3.5 (right side) to pH 10 (left side). Induced CspA is indicated with an arrow.

CspEF30R. With the exception of three genes encoding proteins with the membrane-associated functions CheW, DcuB, and Tap, none of group 3 genes are cold shock inducible. It can therefore be concluded that these genes are probably not the physiological targets of Csp proteins.

The majority of genes in the fourth group are involved in iron transport. These genes are induced in response to overexpression of Csp proteins at 37°C and in the cold-shocked quadruple deletion strain, and some are moderately suppressed in the Δ*cspC* Δ*cspE* strain at 37°C. None of these genes are cold shock-inducible. We do not know the reason for this peculiar behavior, which may not be a direct consequence of Csp proteins function but a response to stress created due to either their overproduction at 37°C or their absence at 15°C.

Since the goal of this study was to identify cellular targets of CspC and CspE, we concentrated on genes that were upregulated by overexpression of CspC or CspE. However, overproduction of Csp proteins also led to repression of some genes. We previously showed that CspE negatively regulates expression of *cspA* at the level of transcription (1). This is likely achieved by direct binding of CspE to the cold box region of *cspA* and increased efficiency of RNAP pausing at a promoter-proximal site (1). This earlier result is supported by both the primer extension data presented in Fig. 1 and microarray analysis, which showed that the *cspA* mRNA was downregulated ~10-fold in cells overexpressing CspE and was upregulated in the Δ*cspC* Δ*cspE* strain (2.5-fold). The inhibition observed upon the CspC overproduction was smaller (2.5-fold) than that observed with CspE. The results of two-di-

mensional gel electrophoresis presented in the lower panel of Fig. 1 also confirm this result, as CspA is hardly detectable in the wild-type cells at 37°C, while in the electrophoregram of proteins from the Δ*cspC* Δ*cspE* strain, a prominent CspA spot is present. Other genes downregulated (two- to threefold) in cells overexpressing either CspC or CspE encoded (i) proteins with membrane-associated functions (*cysA*, *cysU*, *dsdX*, *msyB*, *gspC*, *mhpT*, *nikB*, *tcmA*, and *oppC*), (ii) proteins involved in cell metabolism (*amyA*, *cyoC*, *ddg*, *galE*, *galK*, *galT*, *phoA*, and *talA*), and (iii) diverse proteins (*cbl* and *deaD* [RNA helicase]), including heat shock proteins (*ibpA* and *ibpB*). None of these genes showed a significant change in expression in the Δ*cspC* Δ*cspE* strain, indicating that they are not Csp dependent.

The results presented in Table 1 suggest that most changes in gene expression observed upon overproduction of Csp proteins depend on their nucleic acid melting function, which is strictly correlated with their transcription antitermination function. This finding prompted us to search for the presence of possible *rho*-independent terminators inside and/or in front of genes activated by CspE/CspC overproduction. A bioinformatic search for terminators was performed using a thermodynamic model of transcription elongation that reliably predicts known intrinsic terminators as well as sequences that may stall transcription elongation without causing transcript release (Tadigotla et al., in press). In the model, the free energy (and therefore, stability) of the RNAP elongation complex located at any position along the genomic DNA is defined by three parameters: transcript length, the translocation state of the elongation complex, and the transcription bubble configuration. The advantage of this algorithm is that it is based only on free energy criterion and does not make any assumptions regarding the terminators structure [i.e., a stem-loop followed by a poly(U) track]. We found that several Csp-responsive genes, such as *malEFG*, *citX*, *hdeAB*, *mmuP*, *tnaCAB*, *fruBKA*, *dcu-fumB*, *pyrBI*, *treBC*, *rbsDACBK*, *motAB-cheAW*, *feoAB*, *appY*, *fes-entF-fepE*, *fhuF*, and *sfa* (from Table 1) indeed showed the presence of high-scoring sequences, though most differed from canonical intrinsic terminator structures. We tested one such sequence in an in vitro transcription assay. The sequence, GCGAUGUACAGCAUCGC, is located within the *tnaCAB* operon. The array data showed that *tnaA* and *tnaB* are upregulated by Csp proteins, while *tnaC* is not. The *tnaC* and *tnaA* genes are separated by a gap of 204 nt; the predicted terminator sequence is situated after the gap, in the beginning of *tnaA*. We carried out in vitro transcription using an engineered DNA template that contained the T7 A1 promoter fused to a 100-nt fragment of *E. coli* genome containing the sequence of interest. Stalled *E. coli* RNAP elongation complexes were prepared by nucleotide deprivation and transcription was resumed by the addition of NTPs in the absence (Fig. 2, lane 1), or in the presence of wild-type (lane 2) or mutant (lane 3) CspE. In the absence of wild-type CspE, two major transcription products were seen. Based on their electrophoretic mobilities, one corresponded to 121-nt long runoff transcript, while the other, which was ~70 nt in length, corresponded to a transcription block at the site of predicted element. Experiments involving immobilized transcription complexes revealed, however, that the 70-nt transcript was not released from the solid support (data not shown) and thus apparently corresponded to some kind of arrested complex. As can be seen, the addition of wild-type CspE reduced the intensity of the 70-nt band and increased the amount of the runoff product,

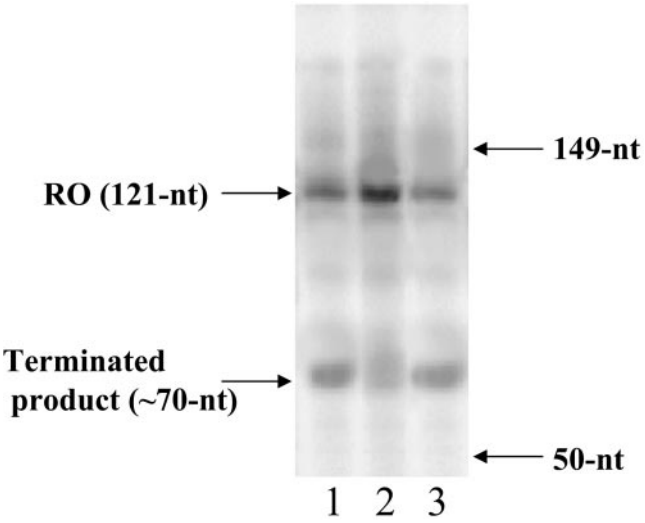

FIG. 2. CspE causes antitermination at the putative transcription terminator in *tnaCAB*. The in vitro transcription assays were carried out as described in Materials and Methods. DNA template containing the T7A1 promoter fused to a 100-nt sequence containing the putative promoter proximal terminator in *tnaCAB* was used and reaction was carried out in absence (lane 1) or presence of wild-type (lane 2) or CspEF30R protein (lane 3). The products were analyzed by urea-polyacrylamide gel electrophoresis (7 M urea–10% polyacrylamide). RO indicates the runoff transcript. *galP1*pDW304- and lambda promoter-containing DNAs were used as control templates to mark the positions of the runoff and terminated transcripts.

apparently recuperating the situation observed in vivo. The mean readthrough efficiency (RE) values, defined as the fraction of the runoff transcripts of the total transcripts produced were calculated for three independent experiments. RE was less than 30% in the control reaction in the absence of CspE, while the addition of wild-type CspE resulted in increase in the RE value to 56%. The RE value in the presence of the mutant CspE, however, was the same as that in the control reaction (no CspE added), indicating that consistent with our previous observations (16), the F30R substitution in CspE abolishes its transcription antitermination activity. Two conclusions can be drawn from this experiment: (i) the sequence that was shown by our bioinformatic analysis to have a potential to act as terminator indeed seems to hinder transcription, and (ii) this effect is reversed by antitermination activity of CspE. This is consistent with the conclusion from Table 1 that majority of genes responding to CspC and CspE depend on their antitermination activity. Additional studies will be necessary to define the exact nature of transcription block exerted by sequences revealed by our search algorithm.

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